Dipeptides Containing L-Arginine Analogs: New Isozyme-Selective Inhibitors of Nitric Oxide Synthase

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Several L-arginine analogs are known as potent inhibitors of nitric oxide synthase (NOS). We recently synthesized dipeptides containing such amino acids, and found that they are potent and isozyme-selective NOS inhibitors. For example, *S*-methyl-L-isothiocitrullinyl-L-phenylalanine showed 66-fold selectivity for iNOS over nNOS, while *S*-methyl-L-isothiocitrullinyl-L-leucine and N^G-nitro-L-argininyl-L-phenylalanine showed 20- and 14-fold selectivity, respectively. Interestingly, *S*-methyl-L-isothiocitrullinyl-D-phenylalanine showed no selectivity, and *S*-methyl-L-isothiocitrullinyl-L-phenylalanine showed competitive inhibition. These results suggest that each NOS isozyme has a cavity of different size near the C-terminal of the L-arginine binding site, and that the selectivity of inhibitors is due to the differences in the size of the cavity.

Key words nitric oxide synthase; selective inhibitor; dipeptide; inducible nitric oxide synthase (iNOS); synthesis; Double-reciprocal plot

The diatomic, short-lived inorganic molecule, nitric oxide (·NO) is a novel biological messenger and immunological regulator.[1] It is synthesized *in vivo* from L-arginine by nitric oxide synthase (NOS). Three isoforms of NOS have been identified. A constitutive form (Type I=nNOS) is found in neuronal tissue and is thought to be a retrograde messenger involved in long-term potentiation.[2] An inducible form (Type II=iNOS) occurs in various immune cells. Cytokine-dependent expression of this isoform leads to release of ·NO in larger, cytotoxic quantities. A second constitutive form (Type III=eNOS) is present in the endothelium and maintains vascular tone *via* ·NO-mediated smooth muscle relaxation. Isoform selectivity is a critical issue in inhibitor design, since inhibition of the endothelial enzyme could lead to undesirable cardiovascular effects. A potent and selective inhibitor of the inducible enzyme would be expected to have wide clinical application, as overexpression of this isoform may potentiate a number of inflammatory conditions or lead to excessive smooth muscle relaxation and, hence, hypotension, a hallmark of septic shock[3]; inhibitors of the neuronal isoform may be useful in stroke[4] and addiction.[5] Some of the most thoroughly studied inhibitors of NOS are shown in Fig. 1. Among them, **1** (N^G-monomethyl-L-arginine, L-NMA) and **2** (N^G-nitro-L-arginine, L-NNA) are structural analogs of L-arginine. They show little or no isozyme selectivity.[6]

NOS has affinity for L-Arg, but not D-Arg, suggesting L-amino acid specificity. Interestingly, a dipeptide of L-Arg, L-Arg-L-Phe is metabolized as well as L-Arg by type II NOS, but poorly by type III NOS.[7] This result indicates that each isoform of NOS has a distinct substrate specificity. Thus, we expected that dipeptides containing arginine analogs might be novel selective inhibitors of NOS isoforms. As amino acid components, L-NNA (**2**), *S*-methyl-L-isothiocitrulline (**3**) and N^δ-iminoethyl-L-ornithine were selected, because they are relatively potent inhibitors among L-Arg analogs.[8]

MATERIALS AND METHODS

General Methods Melting points were determined on a cover glass on an electrothermal melting point apparatus and are uncorrected. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. ^{1}H-NMR and ^{13}C-NMR chemical shifts in $CDCl_3$ are reported relative to internal tetramethylsilane (TMS). ^{1}H-NMR chemical shifts in dimethylsulfoxide (DMSO)-d_6 and D_2O are reported relative to the solvent peak. Fast atom bombardment mass spectrometry was performed on a SX-102 mass spectrometer (JEOL). Silica gel column chromatography was performed with Wakogel C-200 (100—200 mesh) or Merck aluminum oxide 90 F_{254} (70—230 mesh). Thin-layer chromatography was carried out on Merck silica gel 60 F_{254} or Merck aluminum oxide 60 F_{254}.

Synthesis N^δ-(Benzyloxycarbonyl)-N^α-(*tert*-butyloxycabonyl)-L-ornitinyl-L-phenylalanine *tert*-Butyl Ester (**5a**): N^δ-(Benzyloxycarbonyl)-N^α-(*tert*-butoxycabonyl)-L-ornitine (3.70 g, 10 mmol) and L-phenylalanine *tert*-butyl ester (2.43 g, 10 mmol) were dissolved in 35 ml of dichloromethane and cooled (ice bath), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (2.30 g, 12 mmol) was added. The mixture was stirred overnight at room temperature, then was washed with 1% citric acid, 4% $NaHCO_3$, and brine. The solvent was removed *in vacuo* and the product was isolated by column chromatography (silica) using dichloromethane–10% ethyl acetate. Fractions were analyzed for **5a** by TLC (Rf=0.2) using the eluting solvent. Residual solid was recrystallized from hexane–ethyl acetate to afford colorless crystals. The yield was 4.56 g (79%). mp 102 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.34 (m, 5H), 7.3—7.1 (m, 5H), 6.69 (br d, 0.6H), 5.05 (d, 2H), 4.92 (m, 0.7H), 4.70 (q, 1H), 4.21 (m, 1H), 3.35 (m, 1H), 3.15 (m, 1H), 3.06 (dd, 2H, $J_{\beta\beta}$=6.2 Hz, $J_{\alpha\beta}$=1.8 Hz), 1.8—1.5 (m, 4H), 1.43 (s, 9H), 1.38 (s, 9H).

The following compounds were prepared in an analogous

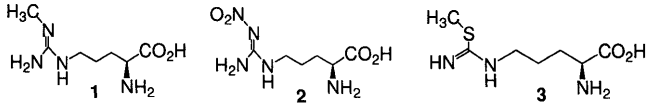

Fig. 1. Structures of L-Arginine Analogs

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manner.

N^{δ}-(Benzyloxycarbonyl)-N^{α}-(*tert*-butyloxycabonyl)-L-ornitinyl-D-phenylalanine *tert*-Butyl Ester (**5b**): Prepared as colorless crystals (4.32 g) in 82% yield; ^{1}H-NMR ($CDCl_3$) δ: 7.3—7.1 (m, 5), 5.59 (br d, 0.7H, J=6.3 Hz), 4.74 (q, 1H, J=7.9 Hz), 4.14 (m, 1H), 3.08 (d, 2H, J=6.4 Hz) 2.67 (m, 2H) 1.8—1.5 (m, 4H) 1.43 (s, 9H), 1.40 (s, 9H).

N^{δ}-(Benzyloxycarbonyl)-N^{α}-(*tert*-butyloxycabonyl)-L-ornitinyl-L-phenylalanine *tert*-Butyl Ester (**5c**): Prepared as a colorless oil (1.74 g) in 78% yield; ^{1}H-NMR ($CDCl_3$) δ: 7.35 (m, 5H), 6.64 (d, 1H, J=9 Hz), 5.15 (m, 1H), 5.09 (d, 2H, J=5.9 Hz), 4.98 (m, 1H), 4.44 (m, 1H), 4.27 (m, 1H), 3.42 (m, 1H), 3.18 (m, 1H), 1.9–1.6 (m, 7H), 1.59 (s, 18H), 0.93 (td, 6H, J=5.2, 2.4 Hz).

N^{α}-BOC-N^{δ}-(Nitro)-L-argininyl-L-phenylalanine *tert*-Butyl Ester (**12a**): Prepared as a pale yellow oil (2.72 g) in 99% yield; ^{1}H-NMR ($CDCl_3$) δ: 8.68 (br s, 1H), 7.94 (br s, 1H), 7.3—7.1 (m, 5H), 4.69 (q, 1H, J=7.0 Hz), 4.21 (m, 1H), 3.30 (m, 2H), 3.11 (dd, 1H, $J_{\beta\beta}$=13.8 Hz, $J_{\alpha\beta}$=6.7 Hz), 3.01 (dd, 1H, $J_{\beta\beta}$=13.8 Hz, $J_{\alpha\beta}$=6.7 Hz)

N^{α}-BOC-N^{δ}-(Nitro)-L-argininyl-L-leucine *tert*-Butyl Ester (**12b**): Prepared as colorless crystals (1.18 g) in 66% yield. ^{1}H-NMR ($CDCl_3$) δ: 7.48 (br s, 1), 6.70 (d, 1H, J=6.0 Hz), 5.39 (d, 1H, J=7.9 Hz, –NH), 4.44 (m, 1H), 4.26 (m, 1H), 3.32 (m, 2H), 1.8—1.5 (m, 7H), 1.46 (s, 9H), 1.44 (s, 9), 0.93 (td, 6H, J=5.5, 1.5 Hz). [FAB-MS] *m/z*: 442 (M^+-44).

N^{δ}-Thioureido-N^{α}-(*tert*-butoxycarbonyl)-L-norvalinyl-L-phenylalanine *tert*-Butyl Ester (**6a**): Compound **5a** (1.71 g, 3 mmol) was dissolved in 10 ml MeOH and stirred at room temperature under argon. Palladium–carbon (10%) was added to the reaction mixture, and the mixture was stirred for 3 h under H_2. After removal of the solvent, the residue was dissolved in 12 ml chloroform and 12 ml water containing 0.40 ml thiophosgene and 0.680 g calcium carbonate. The mixture was stirred vigorously overnight at room temperature. The next day, the reaction mixture was filtered, and the layers were allowed to separate. The aqueous layer was extracted with dichloromethane, and the combined organic layers were dried (Na_2SO_4) and concentrated to an oil by rotary evaporation. The residue was taken up in 24 ml MeOH and cooled to 0 °C. Ammonia gas was passed through the solution for 15 min, and the solution was stirred for 3 h at 0 °C. Following reaction , the solvent was removed, and the residue was chromatographed on silica gel using ethyl acetate–hexane (4 : 1). Product-containing fractions were identified by thin-layer chromatography on a silica gel using the same ethyl acetate–hexane (Rf=0.3). Product-containing fractions were collected and evaporated to dryness under reduced pressure to yield a yellow amorphous solid. The yield was 1.11 g (83%). ^{1}H-NMR ($CDCl_3$) δ: 7.3—7.1 (m, 5H), 6.56 (br s, 0.5H), 6.26 (br s, 0.8H), 5.45 (br d, 1H), 4.67 (q, 1H), 4.15 (m, 1H), 3.54 (m, 1H), 3.07 (dd, 1H), 2.38 (br s, 1H), 1.9—1.5 (m, 4H), 1.42 (s, 9H), 1.39 (s, 9H), ^{13}C-NMR ($CDCl_3$) δ: 171.8, 156.7, 137.0, 130.3, 129.3, 127.9, 83.5, 81.1, 61.3, 54.9, 38.8, 29.2, 28.8, 21.9, 15.1.

The following compounds were prepared in an analogous manner.

N^{δ}-Thioureido-N^{α}-(*tert*-butoxycarbonyl)-L-norvalinyl-D-phenylalanine *tert*-Butyl Ester (**6b**): prepared as a dark red amorphous (1.09 g) in 85% yield; ^{1}H-NMR ($CDCl_3$) δ: 7.3—7.1 (m, 5H), 6.55 (br s, 0.5H, NH), 6.25 (br s, 0.7H, NH), 5.48 (br d, 0.7H, J=7.1 Hz), 4.70 (q, 1H, J=7.0 Hz), 3.53 (m, 1H), 3.14 (m, 1H), 3.07 (qd, 2H, $J_{\beta\beta}$=15.8 Hz, $J_{\alpha\beta}$=7.5 Hz), 2.34 (br s, 1H), 1.41 (s, 18H) [^{13}C-NMR 300 MHz in $CDCl_3$–TMS] δ: 170.8, 156.0 (C=S), 136.1, 128.5, 127.9, 127.9, 82.5, 80.3, 60.4, 55.0, 40.0, 29.1, 24.7, 27.8.

N^{δ}-Thioureido-N^{α}-(*tert*-butoxycarbonyl)-L-norvalinyl-L-leucine *tert*-Butyl Ester (**6c**): prepared as pale yellow oil (0.55 g) in 73% yield; ^{1}H-NMR ($CDCl_3$) δ: 7.08 (d, 1H, J=7.1 Hz), 6.18 (br s, 1H), 5.41 (d, 1H, J=7.5 Hz), 4.41 (q, 1H, J=6.6 Hz), 4.22 (m, 1H), 3.6—3.4 (m, 2H), 1.97 (m, 1H), 1.7—1.5 (m, 6H), 1.46 (s, 9H), 1.43 (s, 9H), 0.93 (t, 6H, J=6.4 Hz).

N^{δ}-(*S*-Methylisothioureido)-L-norvalinyl-L-phenylalanine (**7a**)·2HCl: Compound **6a** (0.59 g, 1.2 mmol) was dissolved in 3 ml acetonitrile. Iodomethane (1 ml) was added to the reaction mixture, and the mixture was stirred at room temperature for 1 h. The solvent was removed, and 3 ml trifluoroacetic acid (TFA), 0.1 ml H_2O, 0.21 g phenol, 0.1 ml thioanisole and 0.05 ml ethanedithiol were added. The mixture was stirred for 30 min at room temperature, and the solvent was evaporated under Ar flash. The gummy residue was washed three times by ether, and dissolved in some water. The solution was applied to an anion exchange resin (Amberlite IRA-400, Cl-form), and eluted with neutral water. The eluent was collected and the solvent was removed by rotary evaporation. The residue was dissolved in methanol, and large quantity of ether was added. The precipitated hygroscopic solid, was collected as an amorphous powder (0.28 g, 55% yield). ^{1}H-NMR (DMSO-d_6) δ: 9.03 (br s, 1H), 7.3—7.2 (m, 5H), 4.42 (q, 1H), 3.85 (t, 1H), 3.09 (dd, 1H), 2.95 (dd, 1H), 2.63 (s, 3H), 1.70 (m, 2H), 1.64 (m, 2H). [FAB-MS] *m/z*: 353 $(M+H)^+$, $[\alpha]_D$=+15.5°/24°C in EtOH. *Anal.* Calcd for $C_{16}H_{24}N_4O_3S\cdot 2CF_3COOH$: C, 41.38%; H, 4.51%; N, 9.65%. Found: C, 41.21%, H, 4.76%; N, 9.67%.

The following compounds were prepared in an analogous manner.

N^{δ}-(*S*-Methylisothioureido)-L-norvalinyl-D-phenylalanine (**7b**): Prepared as colorless crystals (0.258 g) in 42% yield; ^{1}H-NMR (DMSO-d_6) δ: 9.22 (d, 0.5H, J=8.4 Hz), 9.02 (d, 0.5H, J=9.2 Hz), 8.29 (br s, 2H), 7.3—7.2 (m, 5H), 4.55 (m, 1), 3.84 (m, 1H), 3.25 (m, 2H), 2.65 (s, 3H), 1.54 (m, 1H), 1.37 (m, 2H), 1.22 (m, 2H) [FAB-MS] *m/z*: 353 (M^++1).

N^{δ}-(*S*-Methylisothioureido)-L-norvalinyl-L-leucine (**7c**)·2HCl: Prepared as white solid (0.270 g) in 80% yield; ^{1}H-NMR (D_2O) δ: 4.20 (t, 1H, J=7.5 Hz), 3.91 (t, 1H, J=6.3 Hz), 3.28 (t, 2H, J=6.9 Hz), 2.44 (s, 3H), 1.81 (m, 2H), 1.7—1.5 (m, 4H), 1.52 (d, 1H, J=4.6 Hz), 0.77 (dd, 6H, J=10.2, 6.1 Hz). *Anal.* Calcd for $C_{15}H_{38}C_{12}N_4O_6S$ (**7c**·2HCl·EtOH·$2H_2O$): C, 38.05; H, 7.66; N, 11.83. Found: C, 37.87; H, 7.85; N, 11.84.

N^{ε}-Iminoethyl-L-ornitinyl-L-phenylalanine *tert*-Butyl Ester (**9**): Compound **1a** (0.501 g, 0.88 mmol) was dissolved in 5 ml MeOH and stirred at room temperature under argon. Palladium–carbon (10%) (0.051 g) was added to the reaction mixture, and the mixture was stirred for 3 h under H_2. After removal of Pd/C by filtration, the solution was evaporated. The residue and ethyl iminoacetate (0.124 g, 1 mmol) were dissolved in methanol (5 ml) and the mixture was stirred for 12 h at room temperature. Solvent evaporation and alumina column purification (eluent: 6% MeOH/dichloromethane) gave a colorless amorphous solid. 0.173 g (38% yield) ^{1}H-

NMR ($CDCl_3$) δ: 7.27—7.18 (m, 5H, benzene ring), 4.70 (t, 1H, J=5.9 Hz), 4.23 (m, 1H), 3.19 (m, 2H), 3.08 (d, 2H, J=6.6 Hz), 1.99 (s, 3H), 1.9—1.6 (m, 4H), 1.43 (s, 9H), 1.37 (s, 9H).

N^{ε}-Iminoethyl-L-ornitinyl-L-phenylalanine (**10**)·2HCl: N^{ε}-Iminoethyl-L-ornitinyl-L-phenylalanine *tert*-butyl ester (0.173 g, 0.34 mmol) was dissolved in TFA (3 ml) and the solution was stirred for 1 h at room temperature. After removal of the solvent, the gummy residue was washed with ether, and allowed to solidify in HCl-saturated ether. 0.099 g (77% yield) ^{1}H-NMR (D_2O) δ: 7.24—7.15 (m, 5H), 4.60—4.54 (m, 1H), 3.82 (t, 1H, J=6.2 Hz), 3.11 (dd, 1H, $J_{\alpha\alpha}$=15.4 Hz, $J_{\alpha\beta}$=5.5 Hz), 3.08 (t, 2H, J=7.1 Hz), 2.96 (dd, 1H, $J_{\alpha\alpha}$=14.2 Hz, $J_{\alpha\beta}$=8.7 Hz), 2.05 (s, 3H), 1.71 (m, 2H), 1.49 (m, 2H). [FAB-MS] m/z: 321 (M^++1).

The following compounds were prepared in an analogous manner.

N^{δ}-(Nitro)-L-argininyl-L-phenylalanine (**13a**)·0.5H_2O: Prepared as white solid (1.16 g) in 70% yield. ^{1}H-NMR (DMSO-d_6), δ: 7.2—7.1 (m, 5H), 4.31 (dd, 1H, J=9.0, 5.5 Hz), 3.79 (t, 1H, J=6.4 Hz), 3.09 (t, 2H, J=6.8 Hz), 3.06 (dd, 1H, $J_{\alpha\beta}$=5.1 Hz) 2.86 (dd, 1H, $J_{\beta\beta}$=14.2 Hz, $J_{\alpha\beta}$=9.2 Hz), 1.72 (m, 2H), 1.45 (m, 2H). *Anal.* Calcd for $C_{15}H_{23}N_6O_{5.5}$ (**11a**·0.5 H_2O): C, 47.99; H, 6.18; N, 22.38. Found: C, 47.60; H, 6.37; N, 22.09.

N^{δ}-(Nitro)-L-argininyl-L-leucine (**13b**)·TFA: Prepared as white solid (0.460 g) in 79% yield. mp 116—118° ^{1}H-NMR (D_2O) δ: 4.19 (t, 1H, J=6.2 Hz), 3.88 (t, 1H, J=6.2 Hz), 3.15 (m, 2H), 1.78 (q, 2H, J=7.5 Hz), 1.50 (m, 5H), 0.73 (dd, 6H, J=10.4, 6.0 Hz).

N^{δ}-Thioureido-L-norvalinyl-L-phenylalanine (**8a**)·TFA: Prepared as colorless crystals (0.193 g) in 72% yield. ^{1}H-NMR (DMSO-d_6) δ: 8.59 (br s, 1H), 7.28 (br s,1H), 7.3—7.2 (m, 5H), 7.01 (br s, 1H), 4.44 (m, 1H), 3.41 (m, 1H), 2.94 (m, 1H), 1.67 (m, 2H), 1.49 (m, 2H). *Anal.* Calcd for $C_{17}H_{23}F_3N_4O_5S$: C, 45.13; H, 5.12; N, 12.38. Found: C, 45.11; H, 5.42; N, 12.34. $[\alpha]_D$=+32.8°/24 °C in EtOH.

N^{δ}-Thioureido-L-norvalinyl-D-phenylalanine (**8b**)·TFA: Prepared as colorless crystals (0.224 g) in 79% yield. ^{1}H-NMR (DMSO-d_6) δ: 8.66 (br s, 1H), 7.69 (br s, 0.5H), 7.3—7.2 (m, 5H), 7.10 (br s, 1H), 4.52 (m, 1H), 3.69 (m, 1H), 3.14 (d, 1H, J=10.8 Hz), 2.84 (d, 1H, J=9.5 Hz), 1.45 (m, 2H), 1.20 (m, 2H). $[\alpha]_D$=+16.7°/24 °C in EtOH.

Analytical Biochemical Methods Compounds were assayed for the ability to inhibit the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline by a modification of methods previously described.[11—13] Rat neuronal NOS and mouse macrophage NOS were purchased from Cayman. [^{3}H]L-Arginine was from Daiichi Kagaku. L-NNA and Hepes were from Sigma. 6*R*-5,6,7,8-Tetrahydrobiopterin was from Funakoshi. Other reagents (EDTA·2Na, dithiothreitol, NADPH, $CaCl_2$, calmodulin, L-arginine) were from Wako. The cation exchange column employed was Isolute PRS (Uniflex, H^+-form). Enzyme (25 μl) was added to 125 ml 50 mM Hepes, pH 7.4, 1 mM NADPH, 1 mM EDTA, 1 μM dithiothreitol, 6 μM tetrahydrobiopterin, 17 nM [^{3}H]-L-arginine (1.11—2.22 TBq/mol), 1.25 mM $CaCl_2$ (nNOS only), and 10 μg/ml calmodulin (nNOS only). After 5 min incubation at 22 °C, 2 ml 20 mM Hepes (pH 5.5) was added, and the solution was subjected to chromatography on cation exchange resin. The radioactivity of the eluate was counted in a liquid scintillation counter. To determine the kinetic constant, the concentration of L-arginine was varied from 1 to 25 μM and, in this experiment, the incubation time was 20 min. If the enzyme solution contains peptidases, the dipeptides may be cleaved. Therefore, we added several protease inhibitors to the assay mixture (10 mg/ml leupeptin, pepstatin, soybean trypsin inhibitor, 100 mg/ml phenylmethylsulfonyl fluoride).

For each inhibitor, the percentage inhibition was determined at 10 different concentrations. Each observation was performed in duplicate and the replicate values typically varied by <20%. IC_{50} values were determined from the average of the duplicate observations using MacCurveFit. Under these assay conditions, the production of L-citrulline was linear with time for the duration of the experiment.

RESULTS AND DISCUSSION

Several dipeptides which consisted of arginine analogs combined with L-Phe, D-Phe, or L-Leu were prepared in the usual manner. All dipeptides were synthesized from N^{δ}-Z-N^{α}-BOC-L-ornithine (**4**) and the *tert*-butyl ester of L-Phe, D-Phe or L-Leu (Chart 1). The N^{δ}-Z groups of the fully protected products (**5a—c**) were removed with H_2/Pd–C, and the resultant products were treated with thiophosgene and ammoniacal methanol, affording the thiocitrullines **6a—c**. Compounds **6a—c** were *S*-methylated and deprotected with TFA to give **7a—c**. Direct deprotection of **6a**, **b** afforded **8a**, **b**. On the other hand, **5a** was deprotected and treated with ethyl iminoacetate to give the N^{δ}-iminoethyl-L-ornithine (**10**). Protected L-NNA (**11**) and protected L-Phe or L-Leu were combined and deprotected, giving **13a**, **b**. Compounds **7a—c** were converted to the dihydrochloride salts using anion exchange resin.

The IC_{50} value and isozyme-selectivity are shown in Table 1. *S*-Methyl-L-isothiocitrullinyl-L-phenylalanine (**7a**) and *S*-methyl-L-isothiocitrullinyl-L-leucine (**7c**) were potent and selective inhibitors of iNOS. The IC_{50} values of **7a** are 39 μM for nNOS and 0.65 μM for iNOS, indicating 66-fold selectivity for iNOS. Those of **7c** are 20 μM for nNOS and 1 μM for iNOS, indicating 20-fold selectivity for iNOS. Although **3**[12] showed little isozyme-selectivity, dipeptides containing **3** were isozyme-selective. Interestingly, *S*-methyl-L-isothiocitrullinyl-D-phenylalanine (**7b**) showed no isozyme-selectivity and had a lower activity than **3**. Among dipeptides containing L-NNA (**2**), N^{G}-nitro-L-argininyl-L-phenylalanine (**13a**) showed iNOS-selectivity. As **2** is weakly nNOS-selective, it appears that the isozyme selectivity of **2** is reversed by dipeptide-formation. Other compounds failed to show significant activity at 100 μM (data not shown).

The assay mixture contained protease inhibitors (10 mg/ml leupeptin, pepstatin, soybean trypsin inhibitor, 100 mg/ml phenylmethylsulfonyl fluoride), as mentioned above, but no significant difference was observed when these were added or not (Table 2).

Double-reciprocal plots for **7a** are shown in Fig. 2. The results suggest competitive inhibition, with K_i (μM) values of 0.36.[13] Thus the dipeptide appears to bind NOS competitively, and the inhibition is isozyme-selective.

In this investigation, we employed a new approach to design NOS inhibitors: conversion of L-arginine analog amino acids (known NOS inhibitors) to dipeptides. *S*-Methyl-L-

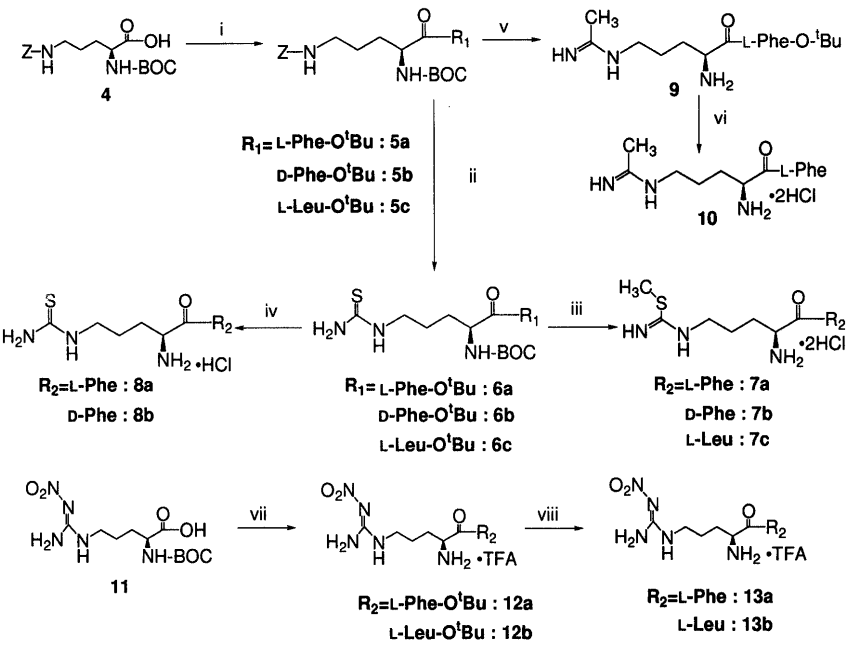

Chart 1. Synthetic Pathway of Dipeptide Compounds

i: H-R_1, WCSI; ii: 1) $CSCl_2$ 2) NH_3; iii: CH_3I 2) TFA 3) ion exchange; iv: 1) TFA 2) ion exchange; v: $CH_3(=NH_2Cl)OEt$; vi: HCl in Et_2O; vii: H-R_2, DCC; viii: TFA.

Table 1. IC_{50} Values and Isozyme-Selectivity

	IC_{50} (μM)		nNOS/iNOS
	nNOS	iNOS	
7a	39	0.59	66
7b	50	47	1.1
7c	20	1	20
13a	>1000	68	>14
3[a]	1.5	0.3	5
2[a]	5	7.5	0.67

Calculated from [^{3}H]citrulline formation. *a*) Known inhibitors.

Table 2. Effect of Protease Inhibitors

Protease inhibitors		(+)	(−)
Control		0.073	0.066
2	10 μM	0.039	0.034
10a	100 μM	0.065	0.055
7a	100 μM	0.027	0.027
7b	100 μM	0.041	0.041

Citrulline formation rate (pmol/min) calculated from [^{3}H]citrulline formation. (+), with protease inhibitors; (−), without.

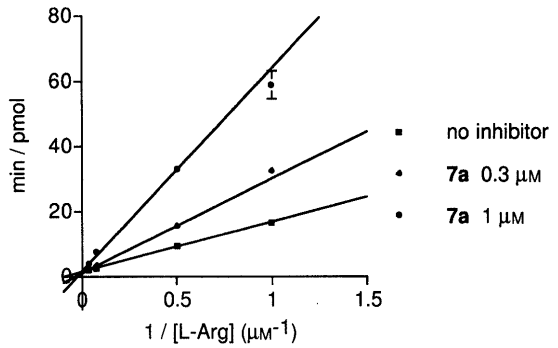

Fig. 2. Plot of the Reciprocal of iNOS Velocity (in Units of min/pmol) *versus* the Reciprocal of L-Arginine Concentration (in Units of μM^{-1}) at Varying Concentrations of **7a**.

NOS activity was determined by measuring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]citrulline as described in the Materials and Methods except that the reaction was initiated by the addition of enzyme and the incubation was carried out at 37 °C for 20 min. Enzyme activity was linear over this time period for each concentration of L-arginine.

isothiocitrulline (**3**, a potent inhibitor) formed dipeptides with L- and D-phenylalanine, and L-leucine. Among the three dipeptides, **7a** was the most potent and selective inhibitor. On the other hand, **7b** was not selective, and was less potent than **7a** and **3**. These results suggest that NOS recognizes the steric environment of amino acids. It is assumed that the inhibitory activity of **3** is caused by the *S*-methylisothiourea group at its side chain and by amino acid recognition by NOS.[14] *S*-Methylisothiourea is a potent inhibitor of NOS.[15] In a recent study, *S*-methyl-D-isothiocitrulline weakly inhibited NOS, presumably because of the *S*-methylisothiourea moiety at its side-chain.[14] Based on that finding, we suggest that the inhibitory activity of [L-, D-] type **7b** is caused by the *S*-methylisothiourea moiety in its side chain but not by recognition of the D-amino acid by NOS. On the other hand, the inhibitory activity of the [L-, L-] type dipeptides may have been largely dependent on amino acid recognition, since this would account for the isozyme selectivity of these compounds. One possibility is that the substrate recognition site of NOS may contain a hydrophobic cavity, the size of which is different in each isozyme. In our investigations, **2** was a weakly selective for nNOS, but the **2**-containing dipeptide, **13a** was selective for iNOS. Other [L-, L-] type dipeptides we prepared were also all iNOS-selective. This result suggests that iNOS has a larger cavity than nNOS. The [L-, L-] type dipeptides have high affinity for iNOS, while the [L-, D-] type dipeptide has low affinity. These results indicate that the assumed hydrophobic cavity is located in a certain direction from the α carbon atom.

Recently, a report of the isozyme-selective NOS inhibitory activity of dipeptides appeared[16] in which the examined

dipeptides consisted of only N^G-nitro-L-arginine and phenylalanine. In contrast, the present report independently describes the inhibitory activity of several combinations of three arginine analogs and three amino acids (L- or D-Phe or L-Leu), thereby showing that arginine analogs and L-amino acid-coupled dipeptides generally have isozyme-selective activity as far as NOS is concerned.

In conclusion, the dipeptides containing L-arginine analogs described here represent a new class of isozyme-selective NOS inhibitors. They may be important as lead compounds for developing new NOS inhibitors, and in elucidating the mechanism of substrate recognition by NOS.

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